

Generation of induced pluripotent stem cells by reprogramming mouse embryonic fibroblasts with a four transcription factor, doxycycline inducible lentiviral transduction system

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SUMMARY

The Stemgent™ DOX Inducible Mouse TF (Transcription Factor) Lentivirus Set was used to reprogram mouse embryonic fibroblasts (MEFs) to an embryonic-like cell state known as the induced pluripotent stem (iPS) cell. These VSV-G-pseudotyped amphotropic lentiviruses are capable of infecting both dividing and non-dividing cells from many mammalian species, including mice and humans. The inducible expression of the mouse transcription factors Oct4, Sox2, Klf4, and c-Myc was made possible by utilizing the doxycycline inducible tetracycline operator (TetO). Co-expression of the four mouse transcription factor-expressing lentiviruses, in combination with the endogenous constitutive expression of reverse tetracycline transcriptional activator (rtTA) transgene, was used to induce reprogramming in mouse embryonic fibroblasts by adding doxycycline (DOX) to the cell culture medium. Pluripotency of the reprogrammed cells was confirmed by pluripotency marker analysis and reporter GFP fluorescence, resulting in a 1.4% reprogramming efficiency, thus demonstrating the utility of the Stemgent™ DOX Inducible Mouse TF Lentivirus Set for the generation of iPS cells from mouse somatic cells.

INTRODUCTION

Reprogramming, the process by which induced pluripotent stem (iPS) cells are generated, is the conversion or “reprogramming” of adult somatic cells to an embryonic-like cell state. Using a defined set of transcription factors and cell culture conditions, Shinya Yamanaka and colleagues demonstrated that retrovirus-mediated delivery and expression of Oct4, Sox2, c-Myc, and Klf4 is capable of inducing pluripotency (i.e. generating iPS cells) in mouse fibroblasts¹. Subsequent reports have demonstrated the utility of the doxycycline inducible lentiviral delivery system for the generation of both primary and secondary iPS cells from a variety of other adult mouse somatic cell types^{2,3}.

Induced pluripotent stem (iPS) cells are similar to embryonic stem (ES) cells in morphology, proliferation, and ability to induce teratoma formation. In mice, pluripotency of iPS cells has been fully demonstrated through the generation of germline chimeras. Furthermore, both ES cells and iPS cells can be used as the pluripotent starting material for the generation of differentiated cells or tissues in regenerative medicine^{4,5,6}. However, iPS cells have a distinct advantage over ES cells as they exhibit key properties of embryonic stem cells without the ethical dilemma of destroying an embryo to obtain the cells. Finally, the generation of patient-specific iPS cells circumvents an important roadblock to personalized regenerative medicine therapies by eliminating the potential for immune rejection of non-autologous transplanted cells⁷.

The ability to reprogram adult somatic cells to iPS cells allows researchers to assess the utility of these cells in a variety of applications. For example, the study of iPS cells generated from diseased and normal patient-specific tissues allow researchers to investigate the cellular mechanisms behind the disease. These iPS cells can be evaluated for their ability to differentiate into a variety of somatic cells. The ability to reprogram adult somatic cells to the iPS cell state also enables the researcher to explore how and why differentiated cell types are able to return to the pluripotent cell state.

Stemgent scientific advisory board member Rudolph Jaenisch, M.D. and his colleagues at the Whitehead Institute for Biomedical Research at M.I.T. developed the doxycycline (DOX) inducible, lentivirus-mediated expression system to regulate the expression of the virally transduced genes^{2,3}. Here we have demonstrated that the Stemgent™ DOX Inducible Mouse TF Lentivirus Set is capable expressing each of the four transcription factors upon transduction into MEFs thereby inducing a pluripotent stem cell state that displays the pluripotency markers characteristic of ES cells.



RESULTS

The Stemgent™ DOX Inducible Mouse TF Lentiviral Set (Cat. No. 00-0003) was used to infect Nanog-GFP/rtTA reporter MEFs in order to demonstrate (1) doxycycline (DOX) regulated expression of the mouse transcription factors, (2) lentivirus mediated transduction efficiency, (3) and reprogramming efficiency. In each experiment in which DOX was added to the growth medium, expression of each of the mouse transcription factors (Oct4, Sox2, Klf4, and c-Myc) was evident when compared to non-DOX induced cells (Figure 1). Lentivirus-mediated transduction efficiency was demonstrated by detecting transcription factor expression by immunocytochemistry (ICC) (Figure 1). Transduction efficiencies of 30%, 40%, 15%, and 15% were achieved for the Oct4, Sox2, Klf4, and c-Myc transcription factors, respectively, demonstrating that these lentiviruses are capable of efficiently expressing the four mouse transcription factors when infecting MEFs.

The over-expression of the four mouse transcription factors correlated well with observed morphological changes that are indicative of the reprogramming process (Figure 2). This morphological transformation progressed over the course of 12 days of DOX treatment to generate larger, more ES cell-like colonies with defined colony edges and three dimensional growth (Figure 3a). Removal of DOX at day 12 resulted in a noticeable reversion of cellular morphology for many of the ES cell-like colonies; however, at day 18, the "iPS" cell colonies that were DOX-independent maintained their ES cell-like morphology and began to express GFP from the endogenous Nanog locus (Figures 3b and 3c). These results demonstrate the utility of the Nanog-GFP/rtTA reporter MEFs as a visible indicator for the initiation of the reprogramming process (Figures 3b and 3c).

The expression of GFP from the endogenous Nanog locus confirmed that the Nanog-GFP/rtTA MEFs were being reprogrammed to generate iPS cells. The iPS cell colonies

in the Day 12 DOX withdrawal dishes continued to increase in size and displayed higher levels of GFP expression. On day 22, forty-seven iPS cell colonies (based on morphology regardless of GFP expression) were manually picked and re-seeded in 24-well plates for expansion. Based on the selection of iPS cell colonies from the three DOX withdrawal dishes, a reprogramming efficiency of 2.3% was obtained (please see below for reprogramming efficiency determination). Of the forty-seven iPS cell colonies, twenty-eight were GFP-positive, giving a GFP based reprogramming efficiency of 1.4%. Subsequent passage of the isolated colonies resulted in the generation of iPS cell colonies that uniformly expressed GFP as well as SSEA1, Nanog, and Oct4 pluripotency markers (Figure 4).

These results demonstrate that the Stemgent™ DOX Inducible Mouse TF Lentivirus Set is efficient for reprogramming experiments; however, several variables should be considered when attempting to optimize the efficiency of reprogramming (i.e. generating iPS cell colonies for further analysis). First, it is possible to modify the active virus-to-target cell ratio (i.e. M.O.I.) during the primary infection step to increase or decrease the transduction efficiency, thereby affecting the number of integrated viruses in the target cell population. Second, adjusting the length of time the cells are exposed to DOX can affect the efficiency of iPS cell colony generation. Third, the proliferative capacity of the target cells can impact reprogramming, as cells which are actively growing and dividing are more amenable to reprogramming⁸. Lastly, when modifying the protocol for different cell numbers or different size tissue culture dishes, it is recommended that target cell numbers are adjusted proportionally to the surface area of the culture dish (please see below for protocol). While no reprogramming method can guarantee reprogramming of any and all cell types, the Stemgent™ DOX Inducible Mouse TF Lentivirus Set (Cat. No. 00-0003) was functionally validated to ensure that all researchers will obtain the best reprogramming results possible.

EXPERIMENTAL PROCEDURES

A. Materials

Cells: Mouse embryonic fibroblasts (MEFs).

Growth Medium (MEF Medium): 450 ml DMEM, 10% FBS, 5 ml 100x non-essential amino acids, 5 ml penicillin (10,000 U/ml)-streptomycin (10,000 µg/ml), 5 ml 200 mM L-glutamine, and 0.5 ml 55 mM β-mercaptoethanol.

Lentiviral Transduction Medium: 29.6 ml of MEF media supplemented with 100 µl mOct4-Lentivirus (Stemgent™ Cat. No. 07-0003), 100 µl mSox2-Lentivirus (Stemgent™ Cat. No. 07-0002), 100 µl mKlf4-Lentivirus (Stemgent™ Cat. No. 07-0005), and 100 µl mc-Myc-Lentivirus (Stemgent™ Cat. No. 07-0004).

EXPERIMENTAL PROCEDURES (CONTINUED)

ES/iPS Cell Medium [DOX (-)]: 450 ml Knockout DMEM, 15% ES cell-qualified fetal calf serum, 5 ml penicillin (10,000 U/ml)-streptomycin (10,000 µg/ml), 5 ml 200 mM L-glutamine, 0.5 ml 55 mM β-mercaptoethanol, and 25 µl 10⁷ U/ml leukemia inhibitory factor (LIF).

B. Seeding MEFs for Reprogramming

Mouse embryonic fibroblasts (passage 2), expressing Nanog-GFP and the rtTA transgene (Nanog-GFP/rtTA MEFs), were seeded at a density of 4 x 10⁵ cells on a single 15 cm cell culture dish coated with 0.2% gelatin. The cells were cultured in 30 ml of MEF medium for 2 days in a tissue culture incubator at 5% CO₂ and 37°C until the desired cell density, approximately 80% confluency (~2 x 10⁶ cells per 15 cm dish), was reached.

C. Viral Transduction

Prior to lentiviral transduction, the MEF growth medium was removed and 30 ml of concentrated lentiviral transduction medium (4 x 100 ml virus stock solution + 29.6 ml MEF medium) was added. After ensuring that the transduction medium was distributed evenly (by gentle rocking of the cell culture dish), the cells were incubated overnight in a tissue culture incubator at 37°C and 5% CO₂.

D. Seeding of Transduced MEFs

Twenty hours post-transduction, the virally transduced cells were trypsinized, centrifuged, and re-suspended in ES cell growth medium for counting and re-seeding (see below for cell densities). The re-seeded cells were incubated overnight in a tissue culture incubator at 37°C and 5% CO₂. (Note: Any remaining transduced cells that were not plated can be frozen in liquid nitrogen for future analysis.) The cells were re-seeded at the following cell densities:

- 2.5 x 10⁵ cells per 10 cm dish; 5 dishes total with 12 ml of ES cell growth medium per dish.
- 2.0 x 10⁴ cells per well of a 4-well plate; 4 plates total with 0.5 ml ES cell growth medium per well.

E. Doxycycline Induced Reprogramming (Day 0)

1. Fresh ES cell medium (DOX (-) medium) was prepared and supplemented with doxycycline at a final concentration of 2 µg/ml to generate the DOX (+) medium.
2. The lentiviral transduction medium was aspirated and replaced with either the DOX (+) or DOX (-) medium as follows below, depending on the experiment:
 - a. For determining reprogramming efficiency: 12 ml medium per 10 cm dish; 4 plates given DOX (+) medium and 1 plate given DOX (-) medium (five 10 cm plates total).
 - b. For determining transduction efficiency by immunocytochemistry: For each 4-well plate, 2 wells were given DOX (+) medium and 2 wells were given DOX (-) medium [0.5 ml medium was added to each well of each 4-well plate (four 4-well plates total)]
3. After replacing the medium, cell culture dishes were placed in a tissue culture incubator at 37°C and 5% CO₂.

F. Medium Changes

1. 10 cm plates: The DOX (+) and DOX (-) medium were replaced every other day.
2. 4-well plates: No medium changes were made. 48 hours (Day 2) post-doxycycline induction, transduction efficiency was determined by immunocytochemistry (please see below).

EXPERIMENTAL PROCEDURES (CONTINUED)

G. Immunocytochemistry – Transduction Efficiency

1. **ICC - Day 1:** Cell staining by ICC was performed individually on a single 4-well plate for each of the 4 transcription factors (Oct4, Klf4, Sox2, and c-Myc) - each plate with two wells of DOX (+) and two wells of DOX (-). One well of each DOX condition was incubated with 1° antibody and 2° antibody, while the other well of each DOX condition served as a negative control (2° antibody incubation only).

- a. Cells were washed once with PBS (without Mg^{2+} or Ca^{2+}).
Note: all subsequent steps require PBS without Mg^{2+} or Ca^{2+} .
- b. Cells were fixed with 0.5 ml 4% paraformaldehyde in PBS per well for 15 minutes at room temperature. The wells were then washed twice with PBS for 5 minutes for each wash.
- c. Cells were permeabilized by adding 500 μ l ice-cold 0.2% Tween®-20 in PBS per well for 10 minutes, then washed twice with PBS for 5 minutes per wash.
- d. Blocking of non-specific binding sites in the cells was performed by adding 200 μ l per well of freshly made 1x non-animal blocking solution and incubating at room temperature for 1 hour.
- e. Cells were then incubated overnight at 4°C with 200 μ l of the primary antibodies diluted with 1x non-animal blocking solution:

Anti-Oct4 diluted 1:100
 Anti-Klf4 diluted to 10 μ g/ml
 Anti-Sox2 diluted to 10 μ g/ml
 Anti-c-Myc diluted 1:100

2. **ICC - Day 2:**

- a. After incubation with the primary antibodies, each well was washed twice with PBS for 10 minutes per wash.
- b. Each well of each 4-well plate was then incubated at room temperature for 1 hour with 200 μ l of the appropriate secondary antibody diluted with 1x non-animal blocking solution:

1° Oct4 → 2° Ab - Donkey anti-Rabbit, Rhodamine conjugate, diluted 1:200
 1° Klf4 → 2° Ab - Donkey anti-Goat, AlexaFluor® 594 conjugate, diluted 1:200
 1° Sox2 → 2° Ab - Donkey anti-Mouse, Rhodamine conjugate, diluted 1:200
 1° c-Myc → 2° Ab - Donkey anti-Rabbit, Rhodamine conjugate, diluted 1:200

- c. Each well was washed twice with PBS for 10 minutes for each wash.
- d. For nuclear staining, each well was incubated with DAPI solution (2 μ g/ml in PBS) for 10 minutes at room temperature and rinsed with PBS.
- e. One to two drops of anti-fade aquamount medium was added to each well to preserve the samples for fluorescence microscopy.

H. Calculating Reprogramming Efficiency and Isolating iPS Cell Colonies

To calculate reprogramming efficiency and to isolate iPS cell colonies, on day 12, the five 10 cm plates (from E.2.a above) were handled as follows: DOX (+) medium was removed from three of the DOX (+) plates and replaced with DOX (-) media. These three DOX withdrawal plates were then labeled as DOX (+/-). This resulted in one plate remaining DOX (-), one plate remaining DOX (+), and three plates converted to DOX (+/-).

EXPERIMENTAL PROCEDURES (CONTINUED)

A. Determining Reprogramming Efficiency

1. Day 2: Transduction efficiency was determined by ICC for each transduced and induced transcription factor, based on the ratio of “positive” cells (i.e. those expressing protein) to the total number of cells. Cells were counted in three independent fields of view at 20x magnification and were averaged to give a final percentage of transduced cells for each transcription factor.
2. Day 22: Using a phase contrast inverted microscope, the colonies were counted in the three DOX (+/-) dishes that exhibited ES/iPS cell colony morphology. Reprogramming efficiency was calculated as follows:
 - A. Percent of total cell count transduced by all 4 viruses = (% Oct4 transduction) x (% Sox2 transduction) x (% Klf4 transduction) x (% c-Myc transduction)
 - B. Number of cells capable of being reprogrammed = (% from step A) x [total cells present in DOX (+/-) from step 1.2]
 - C. Reprogramming Efficiency = (total iPS cell colonies isolated) ÷ (number of cells capable of being reprogrammed)

For example, 250,000 cells were seeded in each of the three 10 cm plates in which doxycycline was withdrawn, giving a total of 750,000 cells capable of being reprogrammed. The transduction efficiencies were as follows: Oct4 = 30%, Sox2 = 40%, Klf4 = 15%, and c-Myc = 15%. Finally, forty-seven iPS cell colonies were selected from the three DOX (+/-) dishes based on morphology. Therefore, the reprogramming efficiency of 2.3% was calculated as follows:

- A. $0.30 \times 0.40 \times 0.15 \times 0.15 = 0.0027$
- B. $0.0027 \times 750,000 = 2,025$
- C. $47 \div 2,025 = 0.023$ or 2.3%

J. Expanding and Characterizing iPS Colonies

1. The forty-seven iPS cell colonies were manually picked and individually trypsinized to dissociate the iPS cell aggregates.
2. Each dissociated colony was re-plated in ES medium in individual wells of a 24-well plate pre-coated with a feeder layer of gamma-irradiated MEFs seeded at a density of 2×10^5 cells/well. The culture medium was changed daily.
3. The 24-well plates were cultured for 6 days in a tissue culture incubator at 37°C and 5% CO₂, and monitored daily for iPS cell colony growth and GFP fluorescence.
4. Individual wells from the 24-well plate that uniformly expressed GFP were trypsinized and passaged 1:8 into two 4-well plates pre-coated with a feeder layer of gamma-irradiated MEFs seeded at a density of 2×10^5 cells/well. Five days later, these plates were then used to analyze pluripotent marker expression by alkaline phosphatase (AP) staining, as well as SSEA-1, Nanog, and Oct4 immunocytochemistry.

K. Alkaline Phosphatase (AP) Staining of Manually Selected and Passaged iPS Cell Colonies (J.4)

1. Alkaline phosphatase (AP) detection was performed according to the manufacturer’s instructions.
2. Cells to be stained were fixed with 4% paraformaldehyde in PBS for 10 minutes and washed twice with PBS for 5 minutes for each wash.
3. The AP substrate solution contained within the kit was added to the appropriate well within the 4-well plate and incubated for 20 minutes for optimum color development and intensity when observed through an inverted microscope.
4. The cells were washed once with PBS and anti-fade aquamount medium was added to each of the wells.

EXPERIMENTAL PROCEDURES (CONTINUED)

L. Immunocytochemistry (ICC) of Manually Selected and Passaged iPS Cell Colonies (J.4)

1. ICC - Day 1

- a. Each well of each 4-well plate was washed twice with PBS for 5 minutes per wash.
- b. Cells were incubated in 0.5 ml of ice-cold 0.1% Triton[®] X-100 in PBS per well for exactly 10 minutes.
- c. After washing three times with PBS (5 min per wash), blocking of non-specific binding sites in the cells was performed by adding 200 μ l per well of freshly made 1x non-animal blocking solution and incubating at room temperature for 1 hour.
- d. Specific individual wells of the 4-well plate were then incubated overnight at 4°C with 200 μ l of primary antibodies diluted with 1x non-animal blocking solution:

Anti-SSEA-1 - diluted 1:200

Anti-Nanog - diluted 1:200

Anti-Oct4 - diluted 1:200

2. ICC - Day 2

- a. Cells were washed twice with PBS for 10 minutes for each wash.
- b. Specific individual wells of the 4-well plate were then incubated at room temperature for 1 hour with 200 μ l of the appropriate secondary antibody diluted with 1x non-animal blocking solution:

1° SSEA-1 → 2° Ab - Donkey anti-Mouse, Rhodamine conjugate, diluted 1:200

1° Nanog → 2° Ab - Donkey anti-Rabbit, Rhodamine conjugate, diluted 1:200

1° Oct4 → 2° Ab - Donkey anti-Rabbit, Rhodamine conjugate, diluted 1:200

- c. After incubation, the cells were washed three times with PBS for 10 minutes for each wash.
- d. The cells were incubated with DAPI nuclear counter staining solution (2 μ g/ml in PBS) at room temperature for 10 minutes.
- e. Each well was rinsed once with PBS and anti-fade aquamount medium was added to all wells before visualization using an inverted fluorescence microscope.

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ADDITIONAL READING

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DATA

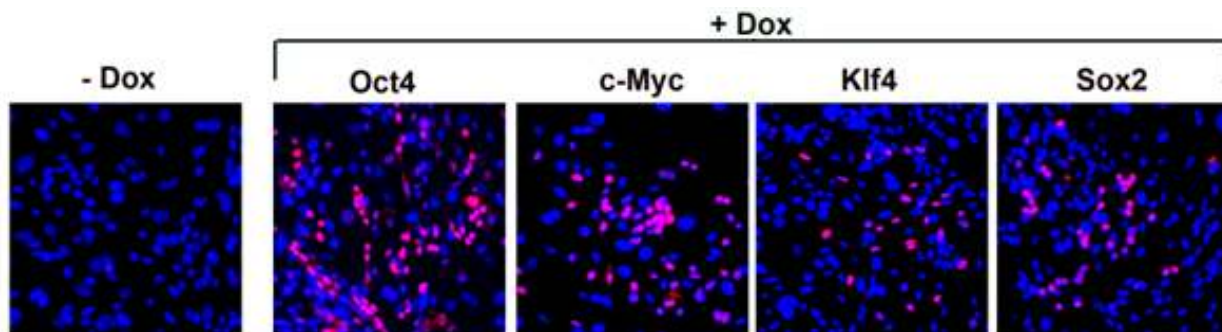


Figure 1. Immunocytochemistry (ICC) analysis 48 hours post-Doxycycline (Dox) induction to monitor transduction efficiency (200x). Nanog-GFP/rtTA mouse embryonic fibroblasts (MEFs) were transduced with each of four lentiviruses from the DOX Inducible Mouse TF Concentrated Lentivirus Set (Cat. No. 00-0003). The Nanog-GFP/rtTA MEFs contain the GFP gene knocked-in at the Nanog locus as well as a reverse tetracycline transcriptional activator (rtTA) expression cassette which is required for DOX inducible expression. The far left panel (-Dox) is a representative negative control for expression of the four transcription factors without DOX induction. Correctly expressed transcription factors were confirmed by corresponding antibodies (shown in red), stained with DAPI to visualize the nucleus.

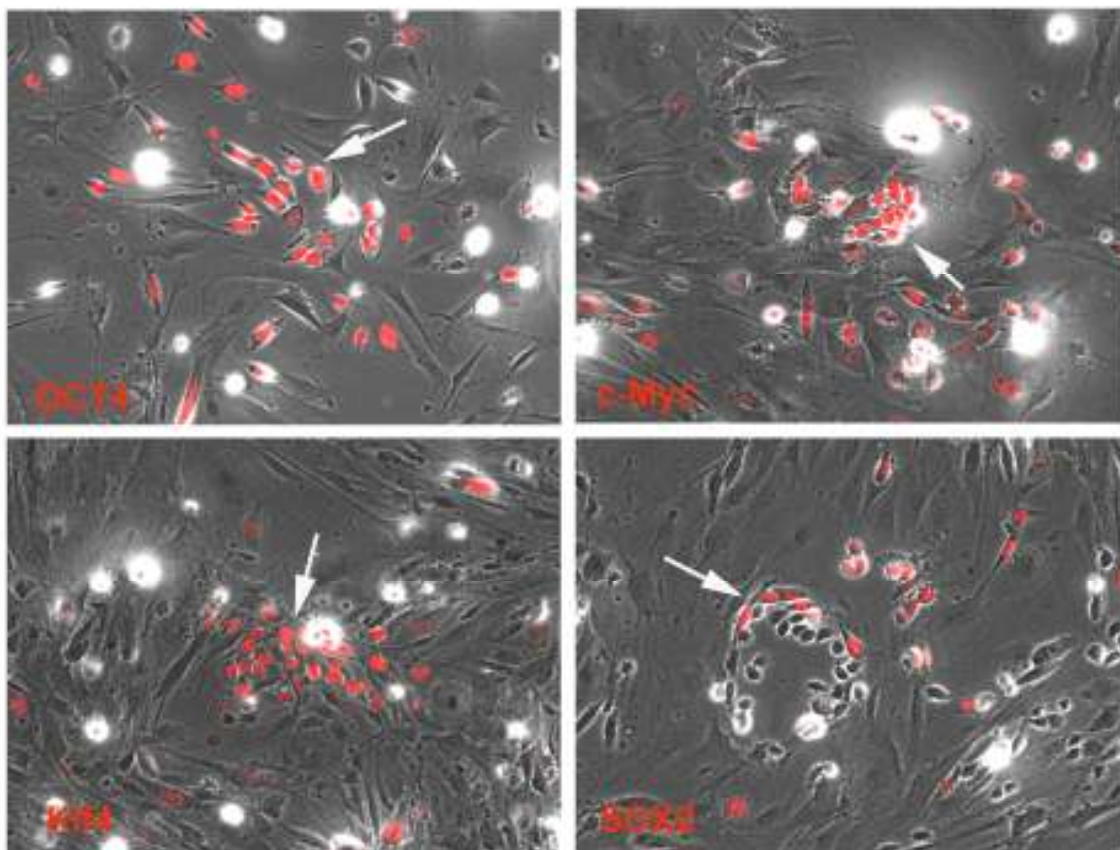


Figure 2. Immunocytochemistry (ICC) analysis 48 hours post-doxycycline (DOX) induction to monitor transcription factor expression associations with morphological changes (200x). Nanog-GFP/rtTA mouse embryonic fibroblasts (MEFs) were transduced with each of four lentiviruses from the DOX Inducible Mouse TF Lentivirus Set (Concentrated) (Cat. No. 00-0003). Cultures were induced with doxycycline (DOX) for 48 hours prior to imaging. Phase-contrast images overlaid with transcription factor specific ICC images (shown in red) to demonstrate localization of expression to morphological changes.

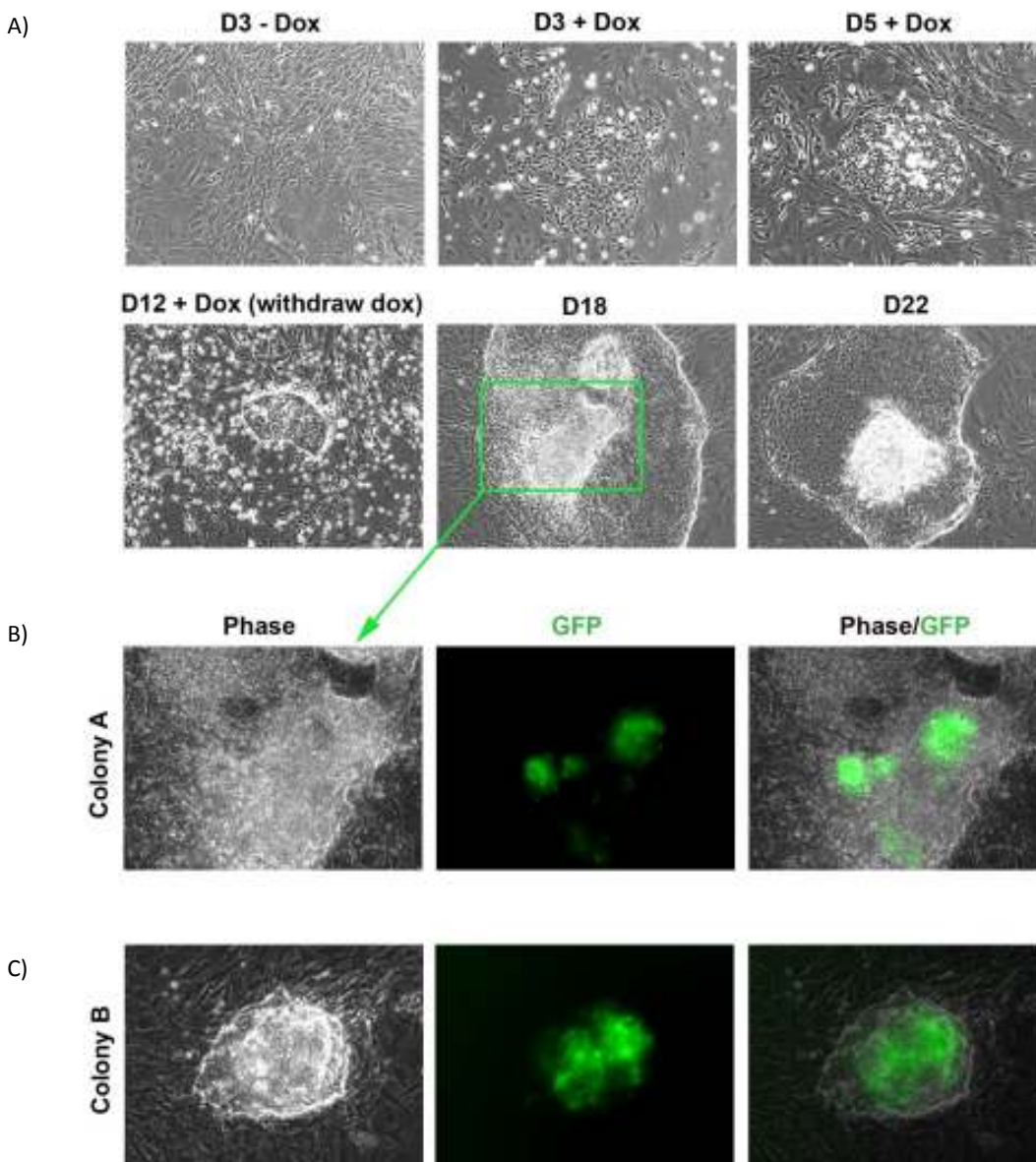


Figure 3. Morphological conversion of Nanog-GFP/rtTA MEFs to the induced Pluripotent Stem (iPS) cell state. Nanog-GFP/rtTA MEFs transduced with all four viruses contained in the DOX Inducible Mouse TF Lentivirus Set (Concentrated) (Cat. No. 00-0003) were visually monitored for changes in morphology and GFP expression following DOX induction. A) 100X phase-contrast imaging of cells from the 100 mm Reprogramming Efficiency and iPS Cell Colony Isolation Dishes demonstrating the compaction and conversion of the Nanog-GFP/rtTA MEFs into iPS cell colonies. DOX removed from 100 mm Reprogramming Efficiency dishes on Day 12. Upper left panel: 20x negative control image from (-) DOX control plate. B) 200x phase-contrast and GFP fluorescence images of highlighted day 18 post-DOX induction iPS cell colony. C) 200x phase-contrast and GFP fluorescence images of additional day 18 post-DOX induction iPS cell colony. GFP expression reflects the endogenous Nanog expression level and is used for monitoring the pluripotent state. GFP fluorescence became visible beginning on day 18 post-DOX induction.

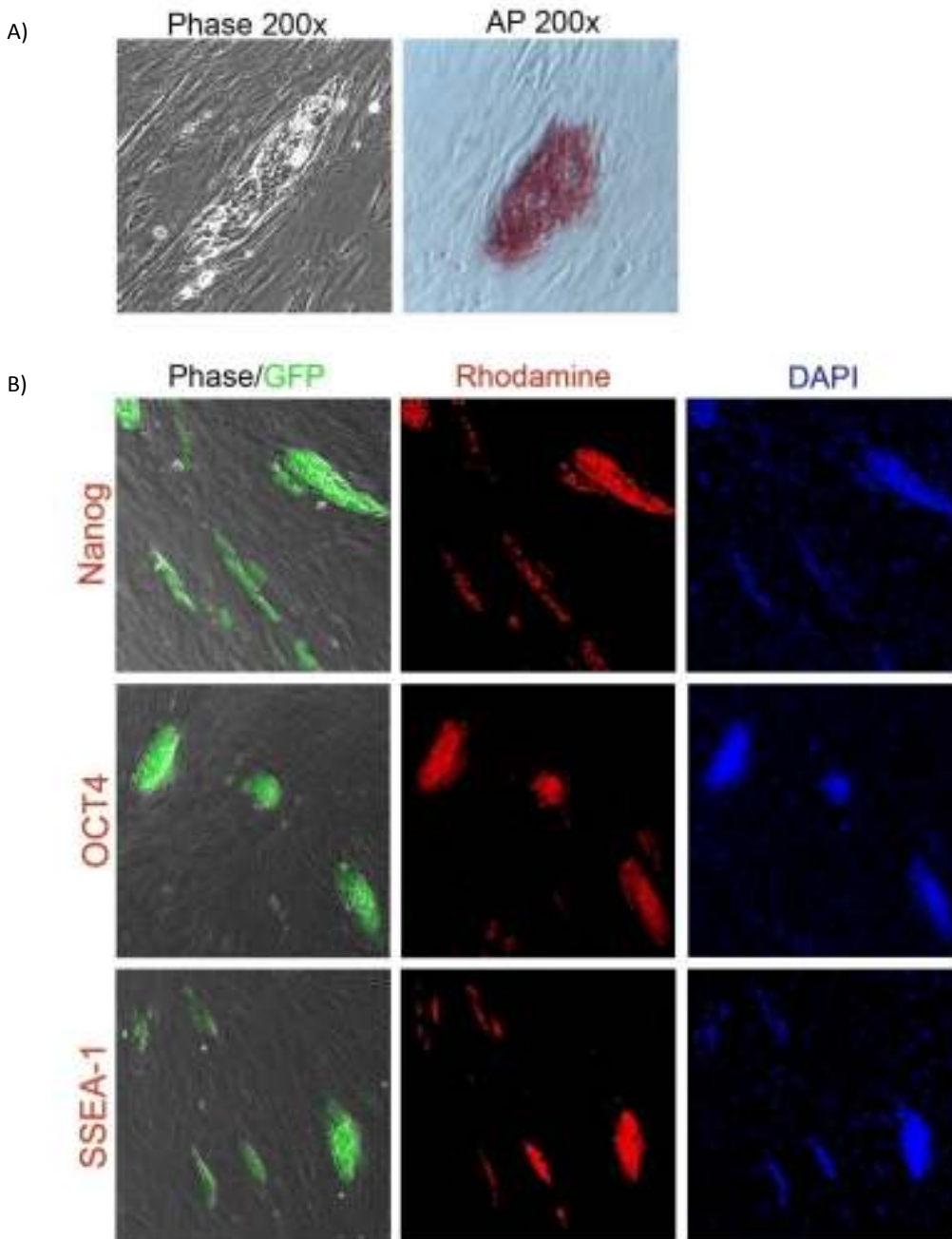


Figure 4. Analysis of iPS cell colonies generated using DOX Inducible Mouse TF Concentrated Lentivirus Set (Cat. No. 00-0003). Nanog-GFP/rtTA MEFs were transduced with all four viruses, carrying Oct4, Sox2, Klf4, and c-Myc cDNA. Expression of the four transcription factors was induced by adding doxycycline and initiating the reprogramming process. GFP expression reflects the endogenous Nanog expression level and is used for monitoring the pluripotent state. The emergent colonies were manually isolated and passaged for further characterization. (A) Phase contrast microscopy and alkaline phosphatase (AP) staining of an induced pluripotent stem (iPS) cell colony (200x). (B) Pluripotency marker analysis (100x): Left Panel - phase contrast overlay with GFP reprogramming reporter expression. Middle Panel - ICC staining for pluripotency marker (Nanog, Oct4, and SSEA1); Right Panel - DAPI staining to visualize the nucleus.